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### Evaluation of immunomodulatory properties of feline mesenchymal stem cells

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1 Title: Evaluation of immunomodulatory properties of feline mesenchymal stem cells

2 Short title: Immunomodulatory properties of feline mesenchymal stem cells

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## 28 **Abstract**

29       Mesenchymal stem cells (MSC) offer a novel approach to treatment of inflammatory  
30 disorders in humans and companion animals. Cats spontaneously develop a wide variety of  
31 inflammatory disorders and may potentially benefit from MSC-based therapies. Multiple  
32 genes are involved in immunomodulation by MSC and interspecies differences between  
33 expressions of these genes exist. The goals of the study were to characterize the expression of  
34 genes known to be involved in MSC-based immunomodulation and determine the effect of  
35 MSC on proliferation of T-cells in co-culture experiments with PBMC. Unstimulated MSC  
36 expressed all immunomodulatory genes studied except for IL-10. Levels of iNOS and FASL  
37 were low or undetectable at the RNA level.  $\text{INF}\gamma$  stimulation resulted in significant dose  
38 dependent upregulation of IDO1, PD-L1, IL-6, COX2 and HGF. Levels of kynurenine were  
39 increased after three-day incubation with  $\text{INF}\gamma$ .  $\text{TNF}\alpha$  stimulation increased expression of IL-  
40 6 at both RNA and protein level as well as upregulated COX2 gene expression and  
41 PTGES1. Stimulation with both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  resulted in significant increase in PGE2  
42 levels in cell culture medium. MSC significantly decreased proliferation of ConA stimulated  
43 PBMC in co-culture experiments at 1:5 ratio. Our results suggest that feline MSC have  
44 similar immunomodulatory gene expression and react to inflammatory cytokines in a manner  
45 similar to human MSC. Thus, MSC may play an important role in treatment of feline disease  
46 as well as serve as an important translational species to evaluate MSC-based therapies of  
47 diseases common to both humans and cats.

48

## Introduction

Mesenchymal stem cells (MSC) have been intensively studied since their first characterization in 1970s [1]. The more recent discovery that MSC are capable of modulating a wide array of immune cells including T-cells, natural killer (NK) cells, dendritic cells, macrophages, B-cells, mast cells and neutrophils [2-9] of and induction of T-regulatory and B-regulatory cells [10,11] has led to interest in their use in patients with inflammatory disorders. MSC-based therapy has been successfully implemented in the settings of both induced inflammatory disorders in rodents,[12,13] as well as spontaneously occurring disorders [11,14,15]. Many questions still remain unanswered such as the optimal route of injection, the dose to be used and diseases that can be targeted. Use of large animal models such as cats may assist in the preclinical phase of investigations for humans. Cats spontaneously develop a variety of inflammatory disorders such as asthma [14], chronic idiopathic (interstitial) cystitis [16], chronic pancreatitis [17], chronic kidney disease [18] or inflammatory bowel disease [19], which can serve as potential translational models for cell based therapies. Many inflammatory mediators have been implicated in immunomodulatory properties of MSC. These factors include indoleamine 2,3 dioxygenase (IDO), inducible nitric oxide synthase (iNOS), prostaglandin E2 (PGE2), interleukin 10 (IL10), hepatocyte growth factor (HGF), transforming growth factor beta (TGF $\beta$ ), interleukin 6 (IL6), programmed death ligand 1 (PD-L1), Fas ligand (FASL), heme-oxygenase 1 (HMOX1) [3,4,8,13,15,20-23]. Interspecies differences exist in immunomodulation between human MSC and murine MSC [20,21,24]. In mice, the immunomodulation is considered to be iNOS dependent [25], while in humans it is IDO1 dependent and iNOS is expressed at very low levels [20,24]. Information on expression of immunomodulatory genes in cats is lacking. Therefore, the current study was undertaken to characterize the immunomodulatory properties of feline MSC.



## Materials and Methods

### *Isolation of feline MSC*

MSC were isolated from adipose tissue of adult cats [20.7 ( $\pm$ 5.7) months] as previously described by our group [26,27]. Tissues were collected from clinically healthy research colony based animals under approved procedures of the Institutional Animal Care and Use Committee at Michigan State University (AUF#: 09/12-171-00). In brief, adipose tissue (2-4 grams) was sterilely excised from subcutaneous tissues, minced and incubated in Collagenase I (Sigma-Aldrich, St.Louis, MO) at a concentration of 1 mg/ml in HBSS (Sigma-Aldrich, St.Louis, MO) for 2 hours at 37°C with 5% CO<sub>2</sub>. The digested tissue was then passed through 70µm cell strainer in order to create single cell suspension and washed in sterile PBS (Sigma-Aldrich, St.Louis, MO). The cell pellet was then suspended in KNAC medium [Keratinocyte SFM medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) supplemented with 2mM of N-acetyl-L-cysteine (Sigma-Aldrich, St.Louis, MO) and 0.2 mM of L-ascorbic acid 2-phosphate (Sigma-Aldrich, St.Louis, MO) ] with 5% of MSC-grade FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and plated in a T25 flask. Cells were trypsinized with 0.05% trypsin after reaching 80% confluency and subsequently passaged using KNAC medium. At each passage, half of the cells were cryopreserved utilizing in Basal Medium (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) supplemented with 20% of FBS (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and 10% DMSO (Sigma-Aldrich, St.Louis, MO). Cells utilized in the experiments described in the manuscript were from passages 3-8. A total of 9 cell lines established from different cats were used interchangeably between the experiments.

### *Phenotypic characterization*

All MSC cell lines were characterized for tri-lineage differentiation as well as expression of CD90, CD44, MHCII and CD105 on cell surface of the cells. Trilineage differentiation was accomplished through utilization of Stempro (Gibco, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) differentiation medium specific for each lineage. Experiments were carried out according to manufacturers recommendations in 24 well plates (USA Scientific, Ocala, FL) for adipogenesis and osteogenesis. For chondrogenesis, instead of using a plate, a 15 ml spinning tube was utilized to form micromass of  $10^6$  number of cells/tube. Cells were incubated in differentiation media for at least two weeks before staining for adipogenesis, utilizing Oil-o-red stain. Two-week incubation period was also used for characterization of chondrogenesis and subsequent Alcian Blue staining. Cells in the osteogenesis group were incubated for 3 weeks and stained with Alizarin red stain.

Expression of cell surface markers CD90, CD44, CD105, MHCII was analyzed with flow cytometry using previously described [28,29] antibodies: CD90 (clone 5E10, BD Biosciences, San Jose, CA), CD44 (clone IM7.8.1, Invitrogen, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY), CD105 (clone SN6, Invitrogen, Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY), MHCII (clone Tu39, BD Biosciences, San Jose, CA). All samples were characterized using LSRII flow cytometer (BD Biosciences, San Jose, CA). Data was then analyzed using commercially available software (FlowJo, LLC, Ashland, OR).

#### Peripheral blood mononuclear cells (*PBMC*) isolation

Peripheral blood was collected into EDTA tubes. Peripheral blood mononuclear cells (PBMC) were isolated using gradient centrifugation with Histopaque-1077 (Sigma-Aldrich, St.Louis, MO) in Accuspin tubes (Sigma-Aldrich, St.Louis, MO) according to manufacturer recommendations. Isolation of feline PBMCs using Histopaque-1077 has been previously described [30]. The cellular interface was collected, washed twice with PBS (Sigma-Aldrich,

123 St.Louis, MO) and cryopreserved in Basal Medium (Gibco, Life Technologies, Thermo  
124 Fisher Scientific Inc, Grand Island, NY) supplemented with 20% of FBS (Gibco, Life  
125 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and 10% DMSO (Sigma-  
126 Aldrich, St.Louis, MO).

### 127 Interferon gamma (*INF $\gamma$* ) and Tumor necrosis factor alpha (*TNF $\alpha$* ) stimulation

128 In order to evaluate the effects of inflammatory cytokines *INF $\gamma$*  and *TNF $\alpha$*  on gene  
129 expression of feline MSC,  $2 \times 10^5$  cells were plated in a 6 well plate in KNAC medium,  
130 allowed to attach overnight and stimulated with increasing concentrations of feline  
131 recombinant *INF $\gamma$*  (R&D Systems, Minneapolis, MN), ranging from 0.25 to 50 ng/mL, or of  
132 feline recombinant *TNF $\alpha$*  (R&D Systems, Minneapolis, MN), ranging from 0.25 to 10 ng/mL.  
133 Cells were incubated for 24 hours for gene expression studies. Both cells and supernatant  
134 were collected and frozen for subsequent analysis at -80° C. For protein/metabolite assays  
135 cells were stimulated for both 24 and 72 hours. In each experiment MSC isolated from 5  
136 different donors were utilized.

### 137 Quantitative Real Time PCR

138 For gene expression analysis, RNA was isolated using mirVana Isolation kit (Life  
139 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY). RNA was quantified on  
140 Qubit 2.0 Fluorometer using Qubit RNA HS assay kit (Life Technologies, Thermo Fisher  
141 Scientific Inc, Grand Island, NY). Quality of RNA was evaluated using Bioanalyzer (Agilent,  
142 Santa Clara, CA) or Caliper LabChip GX (PerkinElmer, Waltham, MA). Only RNA with  
143 RNA integrity number higher than 8 was used for subsequent analyses.

144 Reverse transcription was accomplished using Superscript III reverse transcription kit  
145 (Invitrogen, Thermo Fisher Scientific Inc, Grand Island, NY) and random primer hexamers  
146 (Promega, Fitchburg, WI).

QPCR was performed using custom designed TaqMan primers (Supplemental Table 1; Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and TaqMan Universal Master Mix (Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) on ABI 7900 HT RT-PCR system or StepOnePlus system (Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY). Each assay was run in triplicate. Data analysis was performed using relative quantification method ( $\Delta\Delta$ CT method) [31]. Relative quantification data was log2 transformed (Log2RQ) for normalization and displayed as such in all figures [32].

### *Protein quantification*

IL10 and HGF concentrations were assayed using feline specific ELISA kits (R&D Systems, Minneapolis, MN and MyBioSource, Inc., San Diego, CA respectively). Feline IL6 and multispecies TGF $\beta$ 1 were used to quantify the respective proteins using Milliplex magnetic bead based assays (Millipore Billerica, MA) according to the manufacturers' recommendations. ELISA plates were analyzed in triplicate using a plate reader (PerkinElmer, Waltham, MA), while bead based assays were analyzed in duplicate using Luminex 100 machine (Luminex Corporation, Austin, TX).

### *PGE2 quantification*

A 50- $\mu$ L aliquot of each cell culture medium was transferred to a polypropylene microcentrifuge tube. Then 200  $\mu$ L of methanol with 0.01% BHT (Tokyo Kasei Kogyo Co., Tokyo, Japan) and PGE<sub>2</sub>-*d*<sub>9</sub> (Cayman Chemical, Ann Arbor, MI) as the internal standard was added. After vortexing for 1 min, the mixture was centrifuged at 10,000  $\times$  g for 10 min at 4°C. One hundred  $\mu$ L supernatant was transferred to an amber autosampler vial with 250- $\mu$ L glass insert in preparation for LC-MS/MS analysis. Liquid chromatography separations were

performed using an Ascentis Express C18 column (10 cm×2.1 mm; 2.7 μm particles, Sigma-Aldrich, St. Louis, MO) maintained at 50 °C on a Waters ACQUITY UPLC system (Waters, Milford, MA). The autosampler was cooled to 10 °C, and injection volume was set to 5 μL. Mobile phase A was 0.1% formic acid in water and mobile phase B was acetonitrile. The flow rate of the LC gradient was 0.3 mL/min. The mobile phase gradient began at 1% B, followed by a linear increase to 40% B at 2 min; then to 80% B at 8 min, and 99% B at 9 min, at which the composition was held until 13 min; then returned to 1% B until 15 min. PGE<sub>2</sub> eluted at 4.32 min. The LC column was connected to a Waters Xevo TQ-S triple quadrupole mass spectrometer. PGE<sub>2</sub> and PGE<sub>2</sub>-d<sub>9</sub> were analyzed with electrospray ionization (ESI) multiple reaction monitoring (MRM) in negative-ion mode at transitions of m/z 351.2 → 271 for PGE<sub>2</sub> and 360.2 → 280 for PGE<sub>2</sub>-d<sub>9</sub>. Each transition was monitored with a 0.163s dwell time. The optimized cone voltages of 55 V and 21 V, and collision energy of 16V were selected for both PGE<sub>2</sub> and PGE<sub>2</sub>-d<sub>9</sub>. Other MS parameters were as follows: capillary voltage, 2.5 kV; source temperature, 150 °C; desolvation temperature, 500 °C; desolvation gas flow rate, 600 L/h; cone gas flow rate 150 L/h. Calibration solutions were prepared by serial dilution of PGE<sub>2</sub> (Cayman Chemical, Ann Arbor, MI) stock solution by methanol to concentrations of 20, 4, 0.8, 0.2, 0.05, 0.02, and 0.01 ng/mL. Internal standard PGE<sub>2</sub>-d<sub>9</sub> was added to each calibration solution with the same amount as added in cell culture medium. For quantification, the ratio of PGE<sub>2</sub> peak area to its internal standard peak area was plotted against the concentration, fitted by a weighted (1/x) linear regression using the TargetLynx component of MassLynx 4.1 software (Waters, Milford, MA).

#### *IDO activity measurement*

5×10<sup>5</sup> MSC were plated in a T25 flask in KNAC medium with 5%FBS and allowed to attach overnight. Cell culture medium was then changed to KNAC medium with 5% FBS supplemented with 100μM Tryptophan (Sigma-Aldrich, St.Louis, MO). After a 3 day

196 incubation, the cell culture supernatant was collected and analyzed. To measure the IDO  
197 mediated tryptophan catabolism, a spectrophotometric method was utilized to detect  
198 kynurenine, a stable catabolite of IDO, as previously described [33] with slight modifications.  
199 Cell culture medium was mixed with 30% trichloroacetic acid at a 2:1 ratio, mixed and spun  
200 down at 10000 rpm. Supernatant was then mixed in equal volume with Ehrlich reagent in a  
201 96 well microplate (R&D Systems, Minneapolis, MN). Optical density was then read at 492  
202 nm utilizing a plate reader (PerkinElmer, Waltham, MA).

### 203 *Co-culture experiments*

204 MSC were plated at a density of  $10^4$  cells per well in KNAC medium with 5% FBS  
205 and irradiated with 30Gy using X-RAD320 irradiator (Precision X-ray, North Branford, CT)  
206 in order to stop proliferation. Previous reports have indicated that pre-stimulation with  
207 inflammatory cytokines potentiate the immunomodulatory properties of MSC [20,25]  
208 therefore, we wanted to investigate if that's the case in feline MSC. In order to assess the  
209 effect of prestimulation, cells were prestimulated with the cytokines  $\text{INF}\gamma$  (50 ng/mL) ,  $\text{TNF}\alpha$   
210 (10 ng/mL) or both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  (50 and 10 ng/mL respectively) for 2 hours prior to  
211 addition of PBMCs. Isolated allogenic PBMCs were thawed, washed in PBS. Subsequently  
212 cells were quantified and assessed for viability utilizing Countess cell counter (Life  
213 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY). All PBMCs were  
214 cryopreserved only short-term for 48-72 hours. PBMCs were then suspended in RPMI1640  
215 (Life Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) with 10% FBS (Life  
216 Technologies, Thermo Fisher Scientific Inc, Grand Island, NY) and added at ratio of 1:5 of  
217 MSC to PBMC. Proliferation of lymphocytes was induced by 5 ng/mL of ConA (Sigma-  
218 Aldrich, St.Louis, MO). Cells were pulsed with EdU (Life Technologies, Thermo Fisher  
219 Scientific Inc, Grand Island, NY) 24 hours before the end of the 3-day incubation period.  
220 Fluorescence intensity was then measured using fluorescent plate reader (PerkinElmer,

Waltham, MA). Background fluorescence assessed from wells containing only MSC was subtracted from the total fluorescent values in co-culture wells. Experiments shown represent a single allogenic PBMC donor co cultured with MSC derived from four different donors.

### *Statistical analysis*

Each data set was analyzed for normality using Shapiro-Wilk normality test. Gene expression data was analyzed using ANOVA with post-hoc Dunnett test for normally distributed data, while Friedman test with post hoc Dunn's test was used for non-parametric data. Protein, metabolite and cellular response data were analyzed using t-test or non-parametric Wilcoxon-signed rank test. All analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc, La Jolla, CA)

## **Results**

### *Isolation and characterization of MSC*

All MSC cells isolated strongly expressed the cell surface markers CD90, CD105, CD44 and did not express MHCII (Figure 1). They also underwent trilineage differentiation into chondrocytes, adipocytes and osteocytes as previously described by our group [27] (Figure 2).

### *Quantitative real time PCR*

All cell lines constitutively expressed IDO1, COX2, PTGES1, PTGES2, PTGES3, HGF, TGF $\beta$ 1, IL6, PD-L1, and HMOX1. IL10 was undetectable at RNA level, while levels of iNOS and FASL were low or undetectable. Stimulation with varying concentrations of INF $\gamma$  resulted in increased expression of IDO1 (8.5 to 12.3 Log2-fold increase,  $p < 0.001$ ), which reached plateau at concentrations at 5 ng/ml (Figure 3A). Expression of IL-6 was

upregulated after exposure to  $\text{INF}\gamma$  as well (Figure 3A, 0.3 to 2.1 Log2-fold increase,  $p<0.001$ ), with increased expression being statistically significant at levels as low as 1 ng/ml of cytokine. Concentrations of  $\text{INF}\gamma$  as low as 0.1 ng/ml significantly upregulated expression of PD-L1 (Figure 3A, 1.5 to 3.7 Log2-fold increase,  $p<0.001$ ), while expression of HGF rose gradually with cytokine concentration increment (Figure 3A, 0.29 to 1.8 Log2-fold increase, at 0.25 to 50 ng/ml of  $\text{INF}\gamma$ ,  $p<0.001$ ), with statistically significant result at as low 1 ng/ml of  $\text{INF}\gamma$ . Stimulation with as low as 5 ng/mL of  $\text{INF}\gamma$  resulted in significant increase in COX2 expression compared to unstimulated cells (0.98 to 1.62 Log2-fold increase,  $p<0.05$  to  $p<0.01$ , Figure 3A). Interestingly levels of PTGES1 decreased significantly after stimulation with 50 ng of  $\text{INF}\gamma$  (-0.45 Log2-fold difference,  $p<0.05$ , Figure 3A).

Stimulation of MSC with as low as 0.25 ng/ml of  $\text{TNF}\alpha$  resulted in significantly increased expression of IL6 (Figure 3B, 2.7 to 5.2 Log2-fold difference,  $p<0.001$ ). Expression of COX2 and PTGES1 significantly increased at doses 2.5 and 10 ng/mL of  $\text{TNF}\alpha$  (COX2: 2.08 Log2-fold increase,  $p<0.01$  and 2.014 Log2-fold increase,  $p<0.05$  respectively; PTGES1: 0.758 Log2-fold increase,  $p<0.05$  and 0.8 Log2-fold increase,  $p<0.01$  respectively; Figure 3B).

In order to determine if uPA levels were responsible for decrease of levels of HGF in our protein assay, we investigated uPA levels after stimulation with a single dose of  $\text{INF}\gamma$ . This stimulation resulted in significant upregulation of uPA at gene expression level (2.47 Log2-fold increase,  $p<0.05$ , Figure 3A).

All other genes tested remained unaltered by stimulation with inflammatory cytokines.

In summary, we have identified that 24-hour stimulation  $\text{INF}\gamma$  results in significant dose dependent upregulation of IDO1, IL6, PD-L1, COX2 and uPA, while decreased PTGES1.  $\text{TNF}\alpha$  stimulation resulted in upregulation of COX2 and PTGES1.

*Protein and metabolite quantification*



Levels of IL6 increased after stimulation with each cytokine over 72 hours (TNF $\alpha$ , p<0.01; INF $\gamma$ , p<0.05; both, p<0.001; Figure 4A). Levels of IL6 were also affected by inflammatory environment after 24-hour cytokine stimulation (Supplemental Figure 3).

After 72 hours of stimulation with TNF $\alpha$  amount of TGF $\beta$ 1 significantly increased (p<0.05; Figure 4B), while stimulation with both INF $\gamma$  and TNF $\alpha$  decreased the concentration of secreted TGF $\beta$ 1 as compared to unstimulated cells (p<0.01; Figure 4B).

Interestingly, there were no differences in the concentration of HGF between different conditions at 72 hours, but levels of HGF decreased significantly after three days of incubation compared to day 1 (Supplemental Figure 1), and where on the lower end of detection of the assay.

Levels of PGE2 significantly increased after stimulation of cell with both INF $\gamma$  and TNF $\alpha$  for three days (p<0.05, Figure 5A).

IL10 values were below the detection limit of the only commercially available assay for feline IL10.

### *IDO activity*

INF $\gamma$  stimulation significantly increased kynurenine levels in cell culture supernatants after 3 days of incubation compared to unstimulated cells (p<0.001, Figure 5B).

### *Co-culture experiments*

Co-culture of irradiated MSC with ConA stimulated PBMCs at 1:5 ratio significantly blocked the proliferation of PBMCs as measured by EdU incorporation assay (p<0.001; Figure 6). Prestimulation of MSC with either INF $\gamma$  or with both INF $\gamma$  and TNF $\alpha$  did not change this effect.

## Discussion

The data presented in this study document that feline MSC constitutively express IDO1, PTGES1, PTGES2, PTGES3, HGF, TGF-beta, IL6, PD-L1, HMOX1. Significantly, stimulation with  $\text{INF}\gamma$  for 24hrs, resulted in up to 12- Log2-fold increase in IDO1 expression in MSC, in a manner similar to those observed in human MSC upon stimulation with this cytokine [34]. The upregulation was confirmed through measurement of kynurenine, an IDO1-mediated tryptophan metabolite. The increase in this metabolite was clearly detectable after 3-days of  $\text{INF}\gamma$  incubation. Interestingly, IDO1 is strongly upregulated and functional in human MSC [20,34], whereas in the mouse, this gene is not responsive to  $\text{INF}\gamma$  [3,21,24,35]. Mouse MSC induce iNOS upon stimulation with  $\text{INF}\gamma$ , one of the key molecules involved in murine immunomodulation [25,36]. Expression of iNOS by feline MSC in our experimental conditions was low or undetectable, which closely parallel human MSC [24].

Expression of IL6 was upregulated by both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  stimulation at the RNA level and that was further confirmed by protein assays. Constitutive expression of IL6 is characteristic for MSC [37,38].

FASL and IL10 transcripts were undetectable in the feline MSC and stimulation using  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  did not change this. FASL expression has been recently put forth as being important for MSC mediated T-cell apoptosis [15]. Interestingly the authors of that study did not detect expression of PD-L1 in MSC. However, we have observed strong induction of PD-L1 after stimulation with  $\text{INF}\gamma$ . Our findings are concordant with data reported from placenta derived MSC [39,40], where expression of PD-L1, but not FASL, has been reported. Thus, MSC may use different effectors to induce apoptosis of T-cells. The differences may arise from culture conditions, source of MSC (adipose, placenta, bone-marrow). In previously described studies, bone marrow derived [15,40] and placenta derived cells were utilized [22]. Expression of IL10 by feline MSC was not detectable in our study both at the gene and

protein expression level. At the protein level, the signals generated were below the limit of detection of the commercially available feline IL10 assay (<125 pg/ml). Reports on the production of IL10 by MSC are not concordant. In some studies with human MSC, IL10 was detectable at the gene expression level [41] and at the protein level [22,35], while in others, no IL10 was detected [3,42]. Both the source of MSC and culture conditions may affect the production of IL10.

We have also identified that feline MSC upregulated COX2 in response to both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  stimulation, although all of the PGE2 synthases were strongly expressed in our data set we identified significant increase in PTGES1 expression only after  $\text{TNF}\alpha$  stimulation, while stimulation with  $\text{INF}\gamma$  resulted in significant decrease in expression at the highest dose given. The results on PTGES1 have to be treated with caution as the changes, although statistically significant, may not be biologically effective due to low level of changes. We also identified significant increase in PGE2 levels in cell culture medium after stimulation of MSC with both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$ , while this effect was not identified after stimulation with only a single cytokine. One potential hypothesis would be that increased degradation of PGE2 through upregulation of enzyme responsible for PGE2 degradation HPGD (Hydroxyprostaglandin Dehydrogenase 15-(NAD)). We have investigated this possibility and no increase in expression of HPGD was identified after stimulation with cytokines studied (data not shown). Thus we hypothesize that strong expression of PGE2 synthases in our experimental setting have precluded additional induction through stimulation. Another explanation could be potential species-specific differences in prostaglandin metabolism and further work would need to be done in order to answer this question.

Release of HGF by feline MSC into the supernatant was significantly decreased in all of the conditions the amount of HGF further decreased at 72 hours. In a study with human MSC, 24 hr stimulation with  $\text{INF}\gamma$  was reported to increase HGF release by 10 times [43],

however, the INF  $\gamma$  concentration used was four times higher (200ng/ml) as compared to our study. Thus, different doses of INF $\gamma$  may induce release of HGF at different time points. HGF is being released as an inactive precursor, and in order for it to be fully functional, it requires proteolytic cleavage through urokinase type plasminogen activator (uPA) [44]. In order to investigate if this enzyme is being induced by INF $\gamma$  we performed QPCR analysis of uPA after cytokine stimulation. We found significant 2- Log2-fold induction of uPA after stimulation of MSC with INF $\gamma$  dose used in ELISA studies (50ng/mL). One could speculate that this allows for HGF to be bound by its receptor MET. Interestingly we found that after 3 days of incubation the levels of HGF were hardly detectable in all of the conditions measured, which may suggest that HGF is maybe being cleaved by uPA in all of the conditions.

The levels of TGF $\beta$ 1 were significantly higher after stimulation with TNF $\alpha$  for 3 days, while lower after stimulation with both INF $\gamma$  and TNF $\alpha$  for 3 days. The reason for this disparity is unknown. In one study on human MSC the levels of TGF $\beta$ 1 almost doubled after stimulation with INF $\gamma$  [43]. In another study, stimulation of human MSC with INF $\gamma$  and/or TNF $\alpha$  resulted in decreased production of TGF $\beta$ 1 [45]. Importantly we also identified that TGF $\beta$ 1 is constitutively expressed by feline MSC and TGF $\beta$ 1 was detected as early as after 24 hours of stimulation (Supplemental Figure 2).

Feline adipose derived MSC significantly blocked proliferation of ConA stimulated PBMCs both in naïve state as well as post prestimulation with INF $\gamma$ , TNF $\alpha$  or both. No difference was noted between the prestimulated and naïve cells in our experiments and mixed results have been previously reported in this aspect [25,43,46]. The MSC mediated blockade of ConA PBMC proliferation was previously described [47] and our results are similar to these previously presented.

Significant reduction of stimulated PBMCs seen in co-culture studies described above, along with the gene expression of immunomodulatory molecules documented, provides a

strong rationale for clinical utilization of MSCs in treatment of feline inflammatory disease as well as for the utilization of cats in translational studies.

The current study documents that feline MSC share many similarities in immunomodulatory properties as human MSC and adds to the limited information available from large animal systems. Spontaneously occurring chronic inflammatory disorders in cats and dogs may serve as translational models of MSC-based therapy of human diseases [48]. Studies of MSC in mice have previously reported differences in mice as compared to humans especially with regard to expression of INOS vs IDO [21,24]. The appropriateness of mice models for human immune disorders has recently been challenged [49-51]. Although mouse models will remain important tools in the biomedical field [52], use of spontaneously occurring disorders in cats can potentially successfully fill in the gap between mouse and human in development of new treatment strategies for a variety of disorders[48,53].

The immunomodulatory properties of feline adipose derived MSC documented in this study, provide the rational for the utilization of MSC based approaches to feline diseases with inflammatory components for the treatment of both the feline diseases as well as an informative translational system, valuable for development of human therapies.

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## Author disclosure statement

No competing financial interests exist

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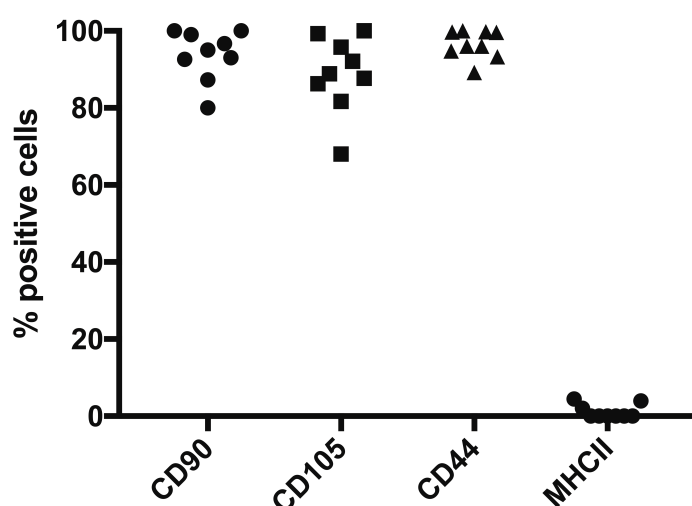
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567

## 568 **Figure legends:**

569 Figure 1. Flow cytometric characteristics of feline MSC used in this study. Note that all of  
570 the cell lines are strongly expressing MSC markers CD90, CD44 and CD105, while lack  
571 expression of MHCII



572

573 Figure 2. Representative pictures presenting differentiation potential of isolated MSC into  
574 adipocytes (A), chondrocytes (B) and osteocytes (C)

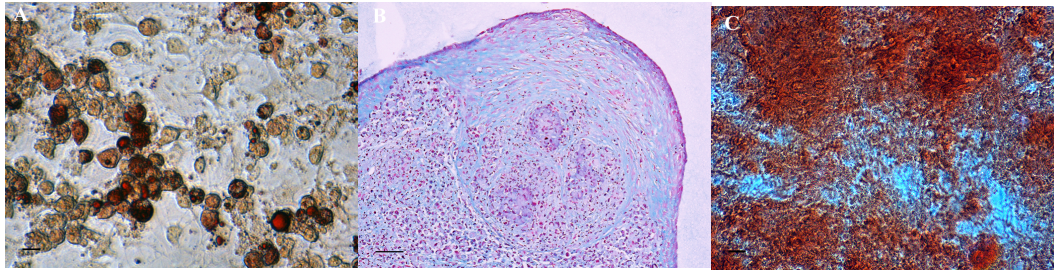
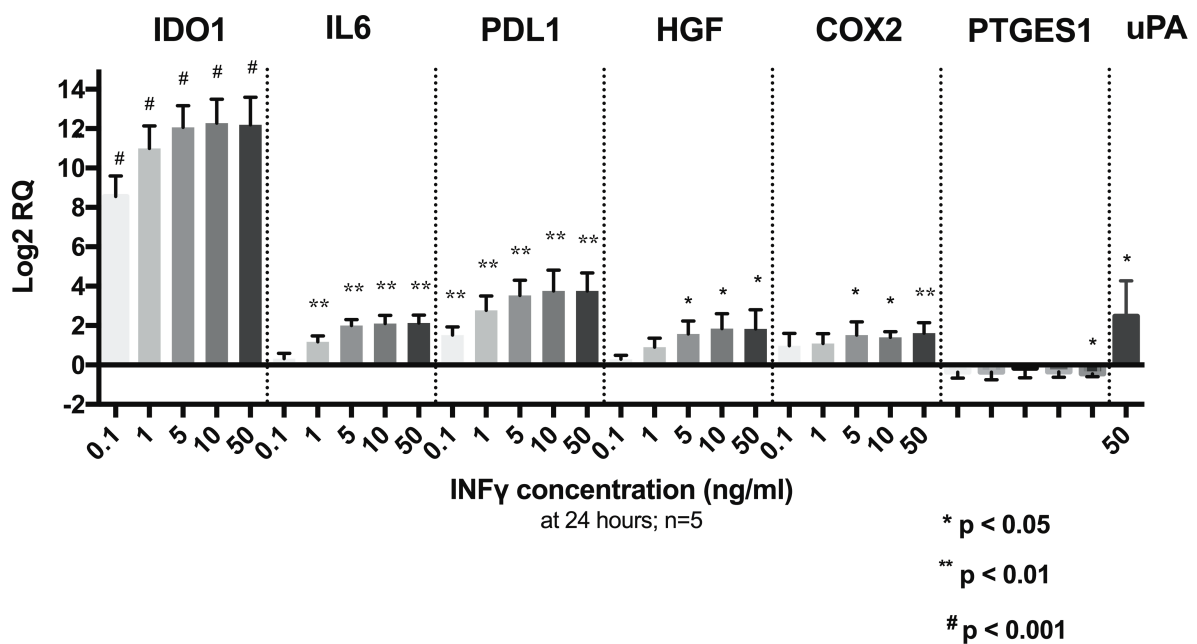


Figure 3. A) Increasing concentrations of  $\text{INF}\gamma$  significantly increased the expression of IDO1, IL6, PD-L1, HGF and COX2 in dose dependent fashion, while decrease in PTGES1 was identified. Stimulation of feline MSC with a single dose of 50 ng/mL of  $\text{INF}\gamma$  results in significant increase in uPA expression; B) Stimulation with  $\text{TNF}\alpha$  significantly increased the expression of IL6, COX2 and PTGES1. n=5

## A



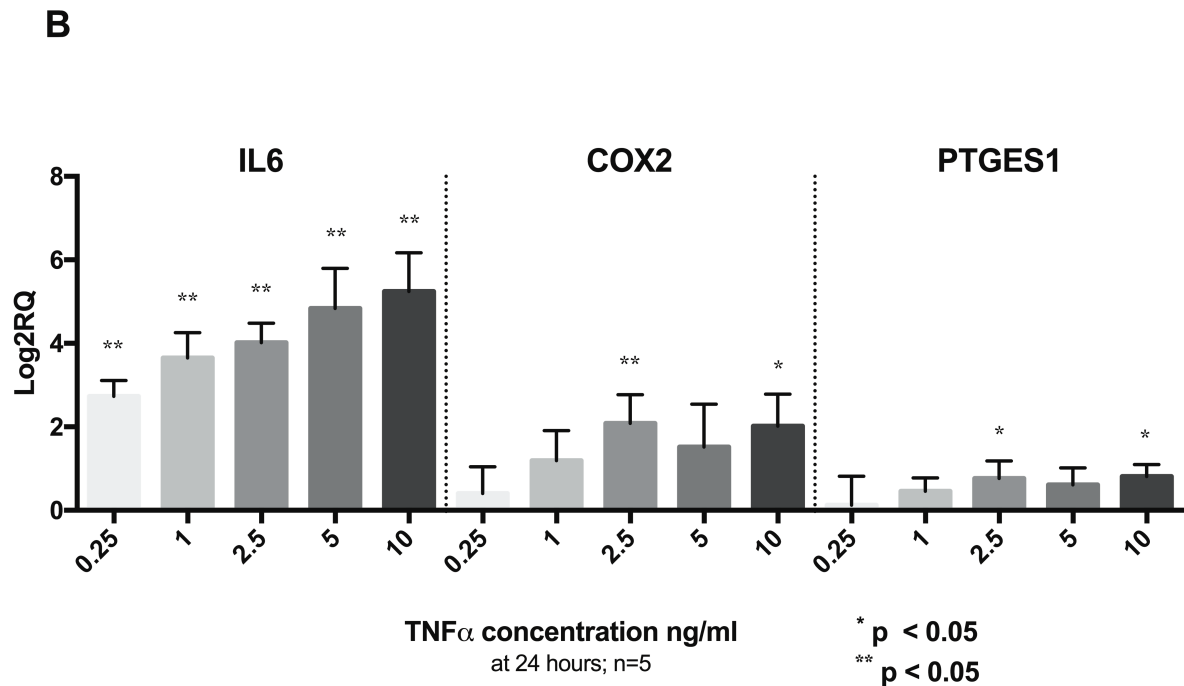
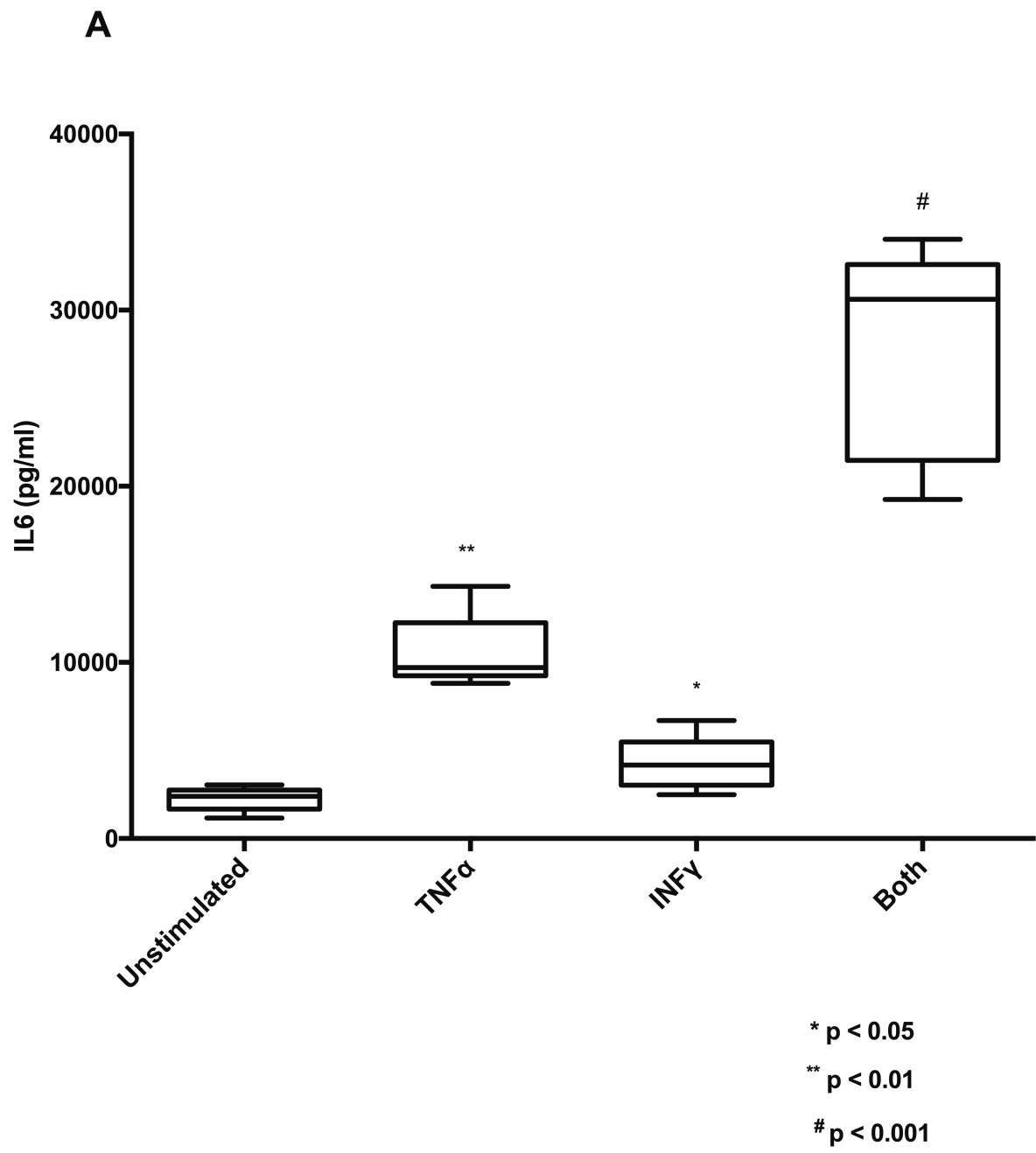
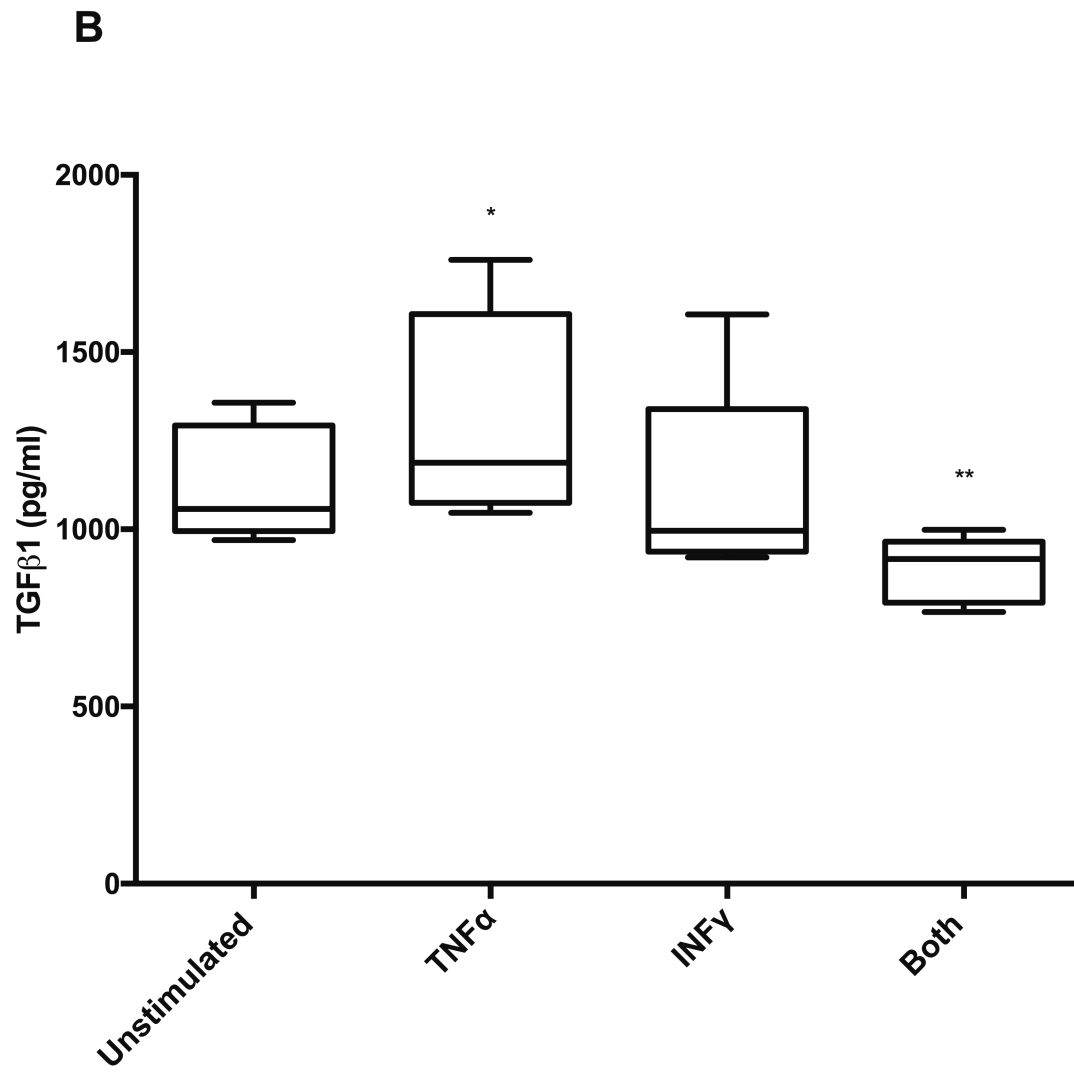


Figure 4. A) IL6 concentration in cell culture medium as measured by ELISA. Note that stimulation with a single dose of TNF $\alpha$  or both TNF $\alpha$  and INF $\gamma$  resulted in significant upregulation of IL6 production; B) Levels of TGF $\beta$ 1 significantly increased after 3-day incubation with TNF and significantly decreased after stimulation with both INF $\gamma$  and TNF $\alpha$ ; C) No changes in HGF expression after 3 days in culture. Interestingly the levels of HGF decreased at 3 days to levels on borderline of detection compared to levels after 1 day (Supplemental materials). n=5 per group, each assay was run in triplicate,







\*  $p < 0.05$

\*\*  $p < 0.01$

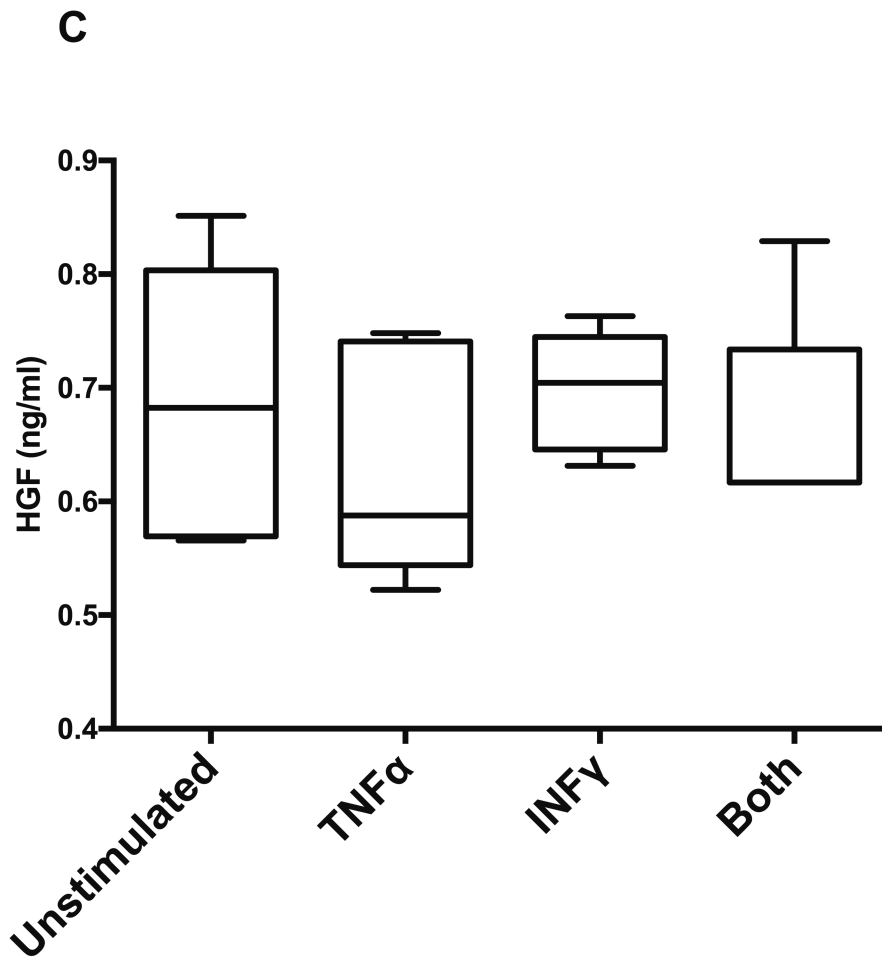


Figure 5. A) Significant increase in PGE2 levels after 3 day stimulation with both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$ ,  $n=5$  ; B) Cells incubated for 3 days with 50 ng/mL of  $\text{INF}\gamma$  in medium supplemented with tryptophan significantly increased the amount of kynurenine in the cell culture medium. $n=3$

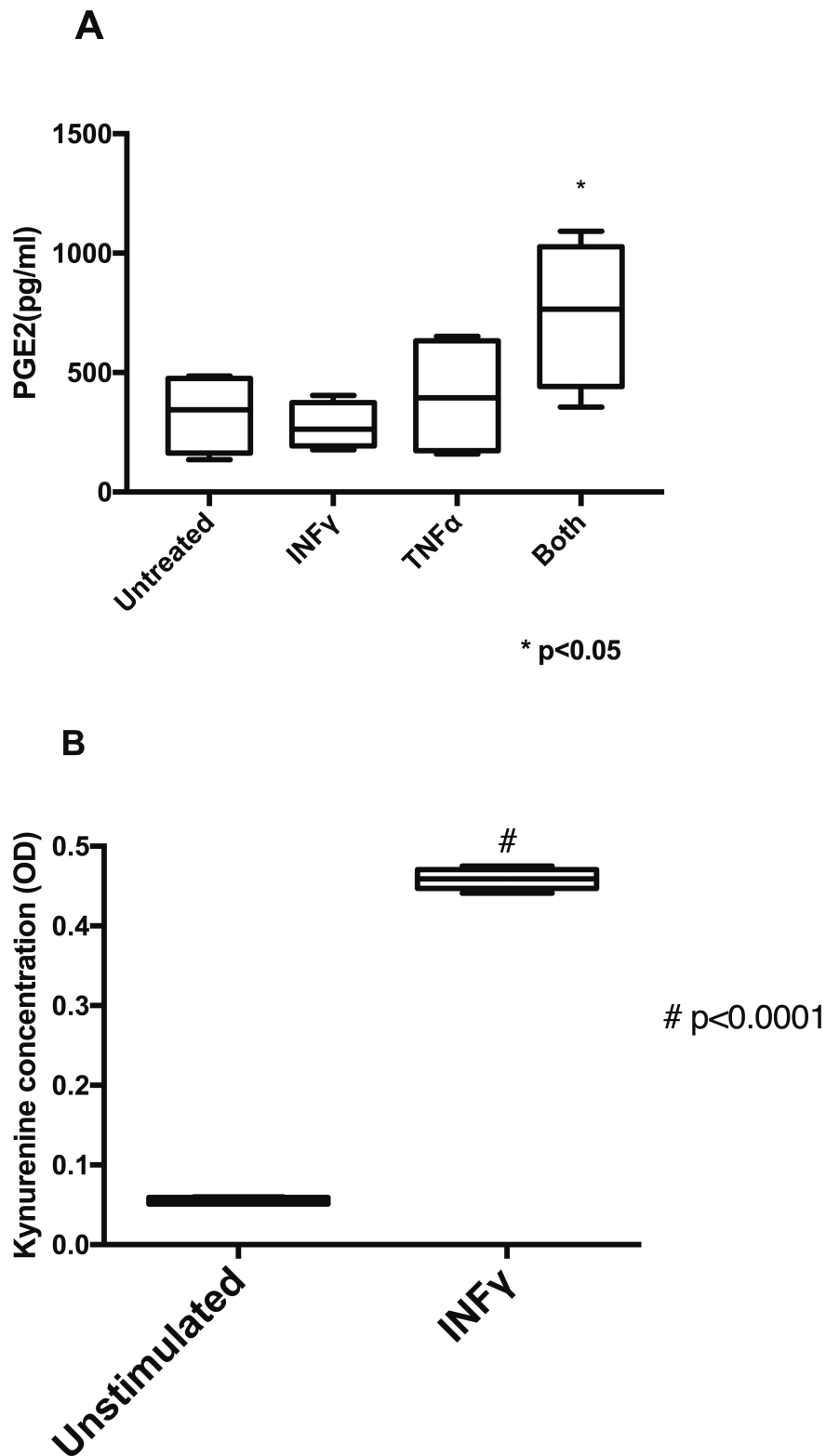
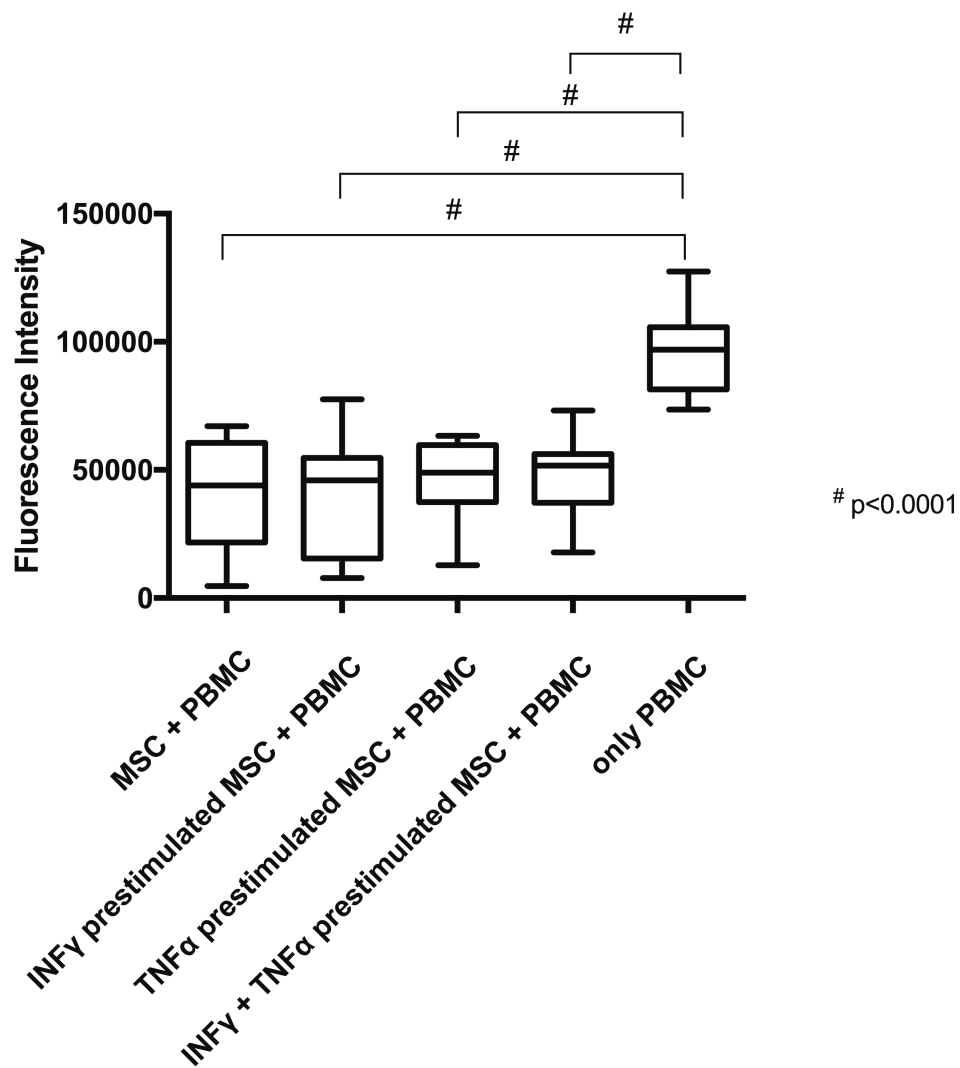


Figure 6. Co-culture experiment revealed a strong decrease in PBMC proliferation at 1 to 5 ratio as measured by fluorescence intensity after subtraction of background proliferation of

601 MSC. No effect of prestimulation with  $\text{INF}\gamma$ ,  $\text{TNF}\alpha$  or both  $\text{INF}\gamma$  and  $\text{TNF}\alpha$  was identified  
602  $n=4$ , each assay was run in triplicate.  $p<0.0001$



603